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Short communication

Oral Angiotensin-(1–7) prevented obesity and hepatic inflammation by inhibition of resistin/TLR4/MAPK/NF-κB in rats fed with high-fat diet

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ABSTRACT

Obesity is characterized by a pro-inflammatory state commonly associated with type 2 diabetes and fat-liver disease. In the last few years, different studies pointed out the role of Angiotensin (Ang)-(1–7) in the metabolic regulation. The aim of the present study was to evaluate the effect of oral-administration of Ang-(1–7) in metabolism and inflammatory state of high-fat feed rats. Twenty-four male Sprague Dawley rats were randomized into three groups: High Fat Diet (HFD); Standard Diet (ST); High Fat Diet + Angiotensin-(1–7) [HFD + Ang-(1–7)]. Glycemic profile was evaluated by glucose tolerance and insulin sensitivity tests, plasmatic glucose and insulin. Cholesterol, HDL and triglycerides analyses presented lipidic profile. RT-PCR evaluated mRNA expression to ACE, ACE2, resistin, TLR4, IL-6, TNF-α and NF-κB genes. The main results showed that oral Ang-(1–7) decreased body weight and abdominal fat-mass. In addition, HFD + Ang-(1–7) treated rats presented enhanced glucose tolerance, insulin-sensitivity and decreased plasma-insulin levels, as well as a significant decrease in circulating lipid levels. These alterations were accompanied by a marked decreased expression of resistin, TLR4, ACE and increased ACE2 expression in liver. Furthermore, Ang-(1–7) decreases phosphorylation of MAPK and increases NF-κB expression. These alterations diminished expression of interleukin-6 and TNF-α, ameliorate inflammatory state in liver. In summary, the present study showed that oral-treatment with Ang-(1–7) in high-fat feed rats improved metabolism down-regulating resistin/TLR4/NF-κB-pathway.

1. Introduction

Obesity is characterized by an increase in white adipose tissue mass, which can result from an excess of food (energy) intake or altered energy expenditure [5]. Obesity has been recently described as a systemic and local adipose proinflammatory state, and this has been implicated in the development of medically important complications, including hepatic steatosis, insulin resistance, and atherosclerosis [16,23,30]. Classic markers of the obesity-induced inflammatory state include the augmented tissue and circulating levels of proinflammatory enzymes, procoagulant factors, cytokines, and chemokines [6,30].

Among these adipokines, resistin is described as a potential factor in obesity-mediated insulin resistance, type 2 diabetes and inflammation [13]. Resistin is a cysteine-rich polypeptide secreted by adipose tissue in rodents and by macrophages in humans, promoting inflammation by regulation of the synthesis and secretion of key proinflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6) in macrophages via a nuclear factor-kappaB-dependent (NF-κB) [24]. Moreover, recent study has provided for the contribution of Toll-like receptor-4 (TLR4) in the pathogenesis of obesity and inflammation [28]. TLR4 and resistin have been linked to a proinflammatory process

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in a human epithelial cell line in which resistin competes with lipopolysaccharide (LPS) for binding to TLR4 [27].

The renin–angiotensin system (RAS) is now recognized to be important for the development of cardiovascular and metabolic disorders [18,20,21]. Angiotensin II (Ang II), a major effector of RAS, is known as a vasoconstrictor, however, recent study has shown its role as a potent mediator in the activation of inflammatory mechanisms involved in obesity [3,26]. On the other hand, angiotensin converting enzyme 2 (ACE2)/Angiotensin-(1–7) (Ang-(1–7))/Mas axis has been suggested as an important counterregulatory arm in the RAS with effects opposite to those of ACE/Ang II/AT1 [18,19]. Ang-(1–7) exerts an important role of antiobesity by Mas receptor [18–21].

The pharmacological potential of Ang-(1–7) was significantly increased after the development of a new oral formulation characterized by a protected Ang-(1–7) molecule included in acyclic-oligosaccharides (cyclodextrin). This novel compound was denominated [hydroxypropyl-β-cyclodextrin/Ang-(1–7) – HPβCD/Ang-(1–7)] [12]. It has been described that Ang-(1–7) included into this HPβCD cavity, can be protected during the passage through the gastrointestinal tract after oral administration [4].

In this context, the aim of the present study was to evaluate the effect of an oral formulation of Ang-(1–7) in diet-induced obesity, metabolic regulation and in resistin liver signaling pathway, which is involved in the inflammation responsiveness.

2. Methods and materials

2.1. Animals

Twenty-four male Sprague Dawley rats from Federal University of the Minas Gerais (Belo Horizonte, Minas Gerais, Brazil) were divided into three groups ($n = 8$) and fed respective experimental diets for 8 weeks: High Fat Diet (HFD), Standard Diet + HPβCD (ST); High Fat Diet + Ang-(1–7) included in HPβCD [HFD + Ang-(1–7)] – 100 μg/kg body weight (dose were corrected ever week according with body weight).

2.2. Diets

The animals fed with Standard Diet (Purina – Labina®) used for regular maintenance of our rats is composed of 50.30% of carbohydrate, 41.90% of protein and 7.80% of fat presenting a total of 2.18 kcal per 1 g of diet. High-fat diet was composed of 24.55% of carbohydrate, 14.47% of protein and 60.98% of fat, presenting a total of 5.28 kcal per 1 g of diet [2].

2.3. Measurements of body weight, food intake and tissue collection

The food intake was measured twice a week during the treatment to obtain food efficiency (food intake/body weight). Overnight fasted rats were killed by decapitation and samples of blood and epididymal, retroperitoneal white adipose tissue and liver were collected, weighed and immediately frozen in dry ice and stored at −80 °C for subsequent analysis.

2.4. Glucose tolerance and insulin sensitivity tests

For the glucose tolerance test, D-glucose (2 mg/g body weight) was intraperitoneally injected into overnight fasted rats. Glucose levels from tail blood samples were monitored at 0, 15, 30, 60, and 120 min. An insulin sensitivity test was performed on overnight-fed rats, after intraperitoneal injection of insulin (0.75 units/kg body weight). Tail blood samples were taken at time 0, 15, 30, and 60 min after injection.

2.5. Determination of plasma parameters

Total serum cholesterol, high-density lipoprotein (HDL), triglycerides were assayed using enzymatic kits (Doles®, Goiania, Brazil). Enzyme-linked immunosorbent assay kits were used to measure serum adiponectin and insulin (Adipo-Gen®, Seoul, Korea) and leptin (Lincoln®, St. Louis, USA) levels.

2.6. Reverse transcription and qRT-PCR

Total RNA from the liver was prepared using TRIzol reagent (Invitrogen Corp.®, San Diego, California, USA), treated with DNAse and reverse transcribed with M-MLV (Invitrogen Corp.®) using random hexamer primers. The endogenous glyceraldehyde 3-phosphate dehydrogenase (GAPDH), ACE, ACE2, resistin, TLR4, IL-6, TNF-α and NF-κB cDNA were amplified using specific primers and SYBR green reagent (Appllied Biosystems®, USA) in an PlusOne platform (Appllied Biosystems®). Relative comparative CT method was applied to compare gene expression levels between groups, using the equation $2^{-\Delta\Delta CT}$ [11].

2.7. Western blot analysis

Proteins were extracted from epididymal adipose tissue samples of rats and 30 μg of protein were resolved on SDS–PAGE gels (10%), transferred onto nitrocellulose membranes and blocked with Odyssey Blocking Buffer 1× (LI-COR Biosciences®, Germany). For immunoblotting, the membranes were probed with a polyclonal rabbit anti-p38/MAPK (Thr180/Tyr182) antibody (1:1000; Cell Signaling Inc., USA). The blots were then incubated with β-actin anti-rabbit IgG (1:1000; Sigma–Aldrich, Germany), was used as endogenous control. The blots were viewed using an infrared Q3127 LICOR® scanner and analyzed using the Odyssey® software. The results were expressed by the relationship antibody primary/β-actin in units of relative density.

2.8. Statistical analysis

Data are expressed as the mean ± SEM. The statistical significance of differences in mean values between rats groups was assessed by one-way ANOVA or 2-way ANOVA (glucose tolerance and insulin sensitivity tests) and the Bonferroni post test. Significance level was set at $P < 0.05$.

3. Results

Oral administration of Ang-(1–7) decreased body weight in HFD + Ang-(1–7) rats when compared with HFD during the period of treatment. At the end of the experiment the body weight was 351.7 ± 17.51 g, 405.0 ± 36.99, and 367.0 ± 35.29 g in ST, HFD and HFD + Ang-(1–7), respectively (Fig. 1A). We did not observe significant alteration between groups when evaluating food efficiency (food intake/body weight) (Fig. 1B).

Analysis of epididymal (ST: 0.0129 ± 0.0039 g/g BW; HFD: 0.0198 ± 0.0031; HFD + Ang-(1–7): 0.0151 ± 0.0034) and retroperitoneal adipose tissues (ST: 0.0098 ± 0.00028 g/g BW; HFD: 0.021 ± 0.0038; HFD + Ang-(1–7): 0.0153 ± 0.0041) demonstrated a reduced fat composition in HFD + Ang-(1–7) (Fig. 1C and D). Additionally, total liver weight g/g BW did not display differences between groups (Fig. 1E).

HFD + Ang-(1–7) rats presented a significant decreased in total cholesterol (ST: 21.62 ± 3.97; HFD: 25.83 ± 3.74; HFD + Ang-(1–7): 20.74 ± 2.72) and triglycerides (ST: 67.88 ± 14.93; HFD:

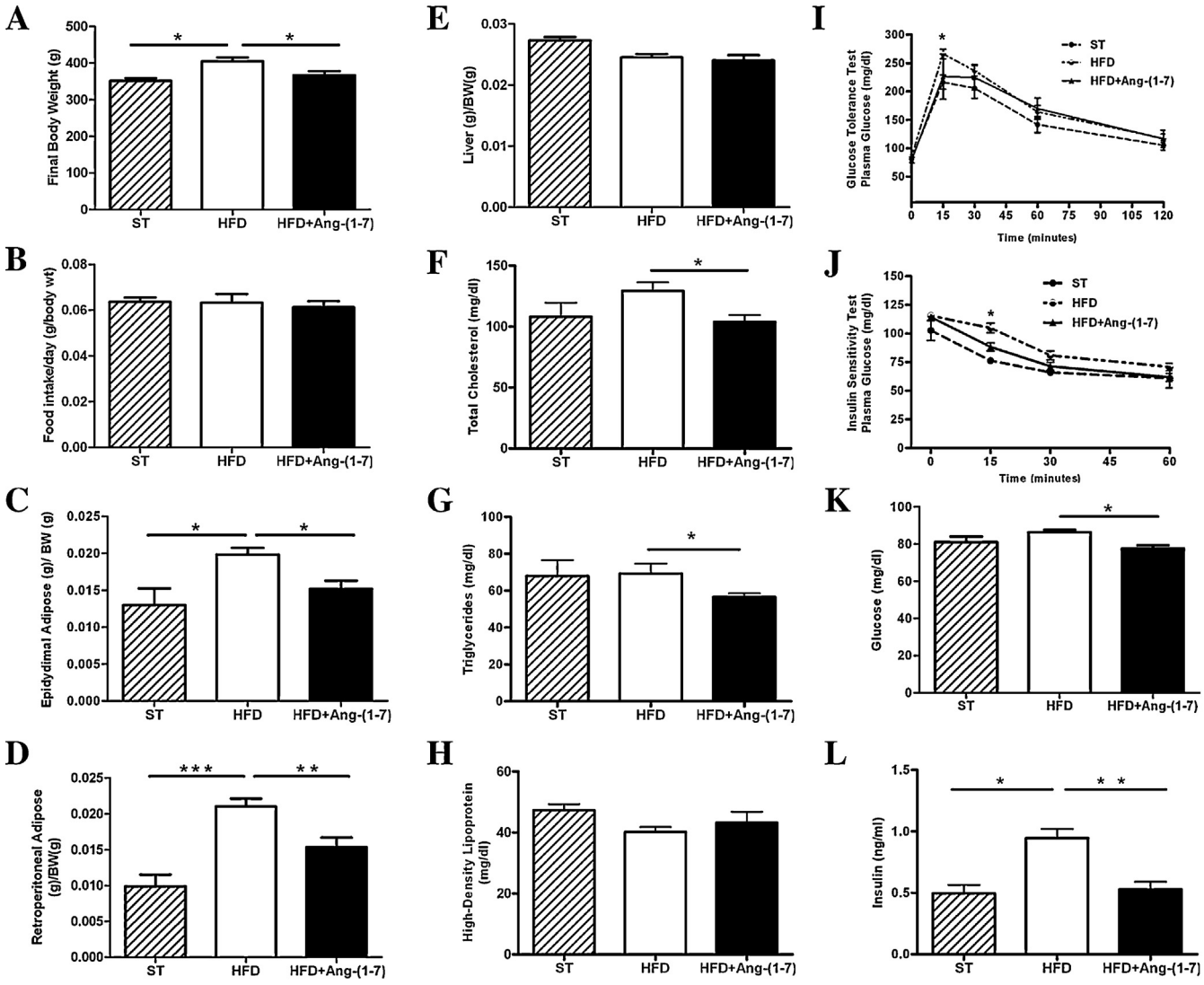

Fig. 1. Body weight, food intake, and lipidic and glycemic profile of ST, HFD and HFD + Ang. (A) Body weight (g) of 8-week-old ST, HFD and HFD + Ang-(1–7) ($n = 8$) male rats. In HFD was significantly increased ($^*P < 0.05$) in comparison to ST and HFD + Ang-(1–7). (B) Food intake, there was no significant difference between the groups. (C) Epididymal adipose tissue weight was significantly increased ($^*P < 0.05$) in comparison to ST and HFD + Ang-(1–7). (D) Retroperitoneal adipose tissue weight was significantly increased ($^{***}P < 0.001$) in comparison to ST and ($^{**}P < 0.01$) in comparison to HFD + Ang-(1–7). (E) Liver weight had no significant difference between the groups. Serum levels of total cholesterol. (F) Serum levels of triglycerides. (G) Serum levels of high-density protein. (H) Glucose tolerance test. Overnight-fasted mice were given an intraperitoneal injection of glucose (2 mg/g body weight). Data are presented as mean of plasma glucose levels (mg/dl) ± SE from six rats in each group. (I) Insulin sensitivity test after intraperitoneal injection of insulin (0.75 units/kg body weight). Blood samples were collected from the tail at indicated time points and analyzed for glucose concentration. Results are expressed as means ± SE from six animals in each group. (J) Serum levels of glucose. (K) Plasmatic levels of insulin. $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ in comparison to the HFD group.

75.97 ± 15.83; HFD + Ang-(1–7): 54.29 ± 4.82) in relation to the HFD group (Fig. 1F and G). Serum levels showed no differences in HDL between groups (Fig. 1H).

A low glucose tolerance and decreased insulin sensitivity were observed in HFD rats when compared with HFD + Ang-(1–7) (Fig. 1I and J). This state was accompanied by a decrease in fasting plasma glucose levels and plasmatic insulin (Fig. 1K and L).

Levels of resistin were significantly higher in HFD rats (ST: 0.79 ± 0.11; HFD: 1.08 ± 0.16; HFD + Ang-(1–7): 0.63 ± 0.18) (Fig. 2A). Additionally, we examined the effect of Ang-(1–7) treatment on TLR4 expression. Our data showed that HFD + Ang-(1–7) rats markedly decreased the mRNA expression of TLR4 in the liver (Fig. 2B).

To investigate the potential link between resistin and proinflammatory pathways, we studied the impact of oral of Ang-(1–7) treatment in rats on the phosphorylation of mitogen-activated protein kinase (MAPK), levels of resistin/TLR4-signaling components and proinflammatory cytokines in the livers of these animals. HFD + Ang-(1–7) group showed decreased total and phosphorylation MAPK expression as compared with the HFD group (Fig. 2C and D). Additionally, this study revealed increased ACE2 and decreased ACE expression (Fig. 2E and F). We did not observe significant alteration between groups when evaluating Mas receptor expression (Fig. 2G).

The mRNA expression of proinflammatory cytokines by q RT-PCR in the liver showed a significant decrease of NF-κB, TNF-α and IL-6 in HFD + Ang-(1–7) group (Fig. 3A and C). The expression of the

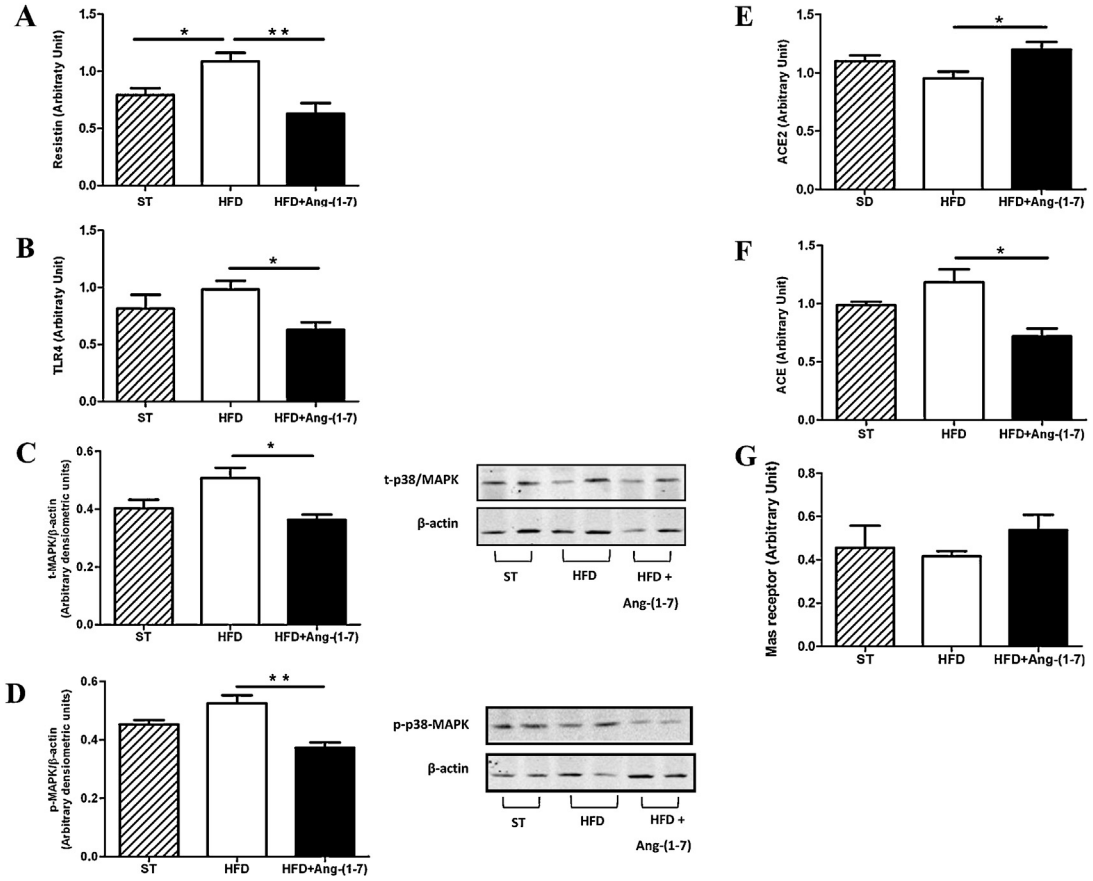

Fig. 2. Liver mRNA expression performed by qRT-PCR and western blotting analyses. (A) mRNA expression of resistin. (B) mRNA expression of TLR4. (C) Western blot for total MAPK protein. Illustrative picture of western blotting gel. (D) Western blot for phosphorylated MAPK protein. Illustrative picture of western blotting gel. After incubation with primary and secondary antibodies for p38/MAPK detection, the membrane was stripped and incubated with primary and secondary antibody used to detect β-actin. (E) Expression of ACE2. (F) Expression of ACE. (G) Expression of Mas receptor. $^{*}P < 0.05$ and $^{**}P < 0.01$ in comparison to the HFD group.

IL-1β did not differ among the groups (Fig. 3D). Fig. 3E illustrates signaling mechanism involved Resistin/TLR4/MAPK/NF-κB.

4. Discussion

Obesity is a pernicious public health problem commonly associated with type 2 diabetes and insulin resistance state. Recent studies linked different obesity complications and insulin resistance state with high resistin levels [13]. Resistin is an important adipokine that is positively correlated with high-fat mass and has been associated with a proinflammatory state as reported in chronic liver diseases [16]. Resistin also modulates the synthesis and secretion of key proinflammatory cytokines such as TNF-α and IL-6 through a NF-κB-dependent pathway [17]. Despite of several recent studies describing resistin pathophysiology, only a small part of resisitin signaling is known and its importance in inflammation process has just started to be investigated. In the present study we evaluated for the first time the effects of oral Angiotensin-(1–7) administration in the inhibition of the inflammatory pathway – resistin/TLR4/MAPK/NF-κB in the liver of obese rats.

Recent studies demonstrated the benefit of metabolic effects of the Ang-(1–7)/Mas axis activation [2,19–21]. In the present study we mainly observed that oral formulation of Ang-(1–7) produced an important reduction in body weight and adipose tissue mass associated with decreased serum total cholesterol and triglycerides levels followed by ameliorated insulin sensitivity, glucose tolerance and diminished expression of proinflammatory cytokine mRNAs. Additionally, we showed a decrease in TLR4 and MAPK expression in the liver associated with decreased ACE and increased ACE2 expression.

Liver is a complex and important organ and plays an essential role on lipid and glucose metabolic regulation. Several studies showed that many RAS components are expressed in the liver meddling metabolic and inflammatory processes [2,29]. The increased expression of Ang II induced non-alcoholic fatty liver disease and modulates inflammatory cell recruitment into the liver during liver injury [8,29]. Additionally, it was previously demonstrated that ACE2/Ang-(1–7)/Mas axis expression is down-regulated during obesity [20,21]. Rats with increased Ang-(1–7) levels had lower body weight and decreased IL-1β and COX-2 in adipose tissue associated with improved liver glucose metabolism [2]. Our results are in agreement with these data showing an elevated expression of ACE2 and decreased ACE in the livers of HFD + Ang-(1–7) treated rats.

It has been shown that lipid and glycemic parameters can be modulated by resistin expression. [22], especially considering that resistin is produced by adipocytes, which are augmented in obese liver. Kushiyama et al. showed that resistin-like molecule beta activates MAPKs, suppresses insulin signaling in hepatocytes and induces diabetes, hyperlipidemia and fatty liver in transgenic mice on a high fat diet model [9]. Our results indicate that obese rats with increased liver resistin expression exhibit an insulin-resistant state, glucose intolerance, hyperinsulinemia and altered lipid metabolism. Furthermore, we showed that rats treated with Ang-(1–7) presented with diminished liver resistin expression associated with increased ACE2 expression. These results are in agreement with the data obtained in previous studies [9,10].

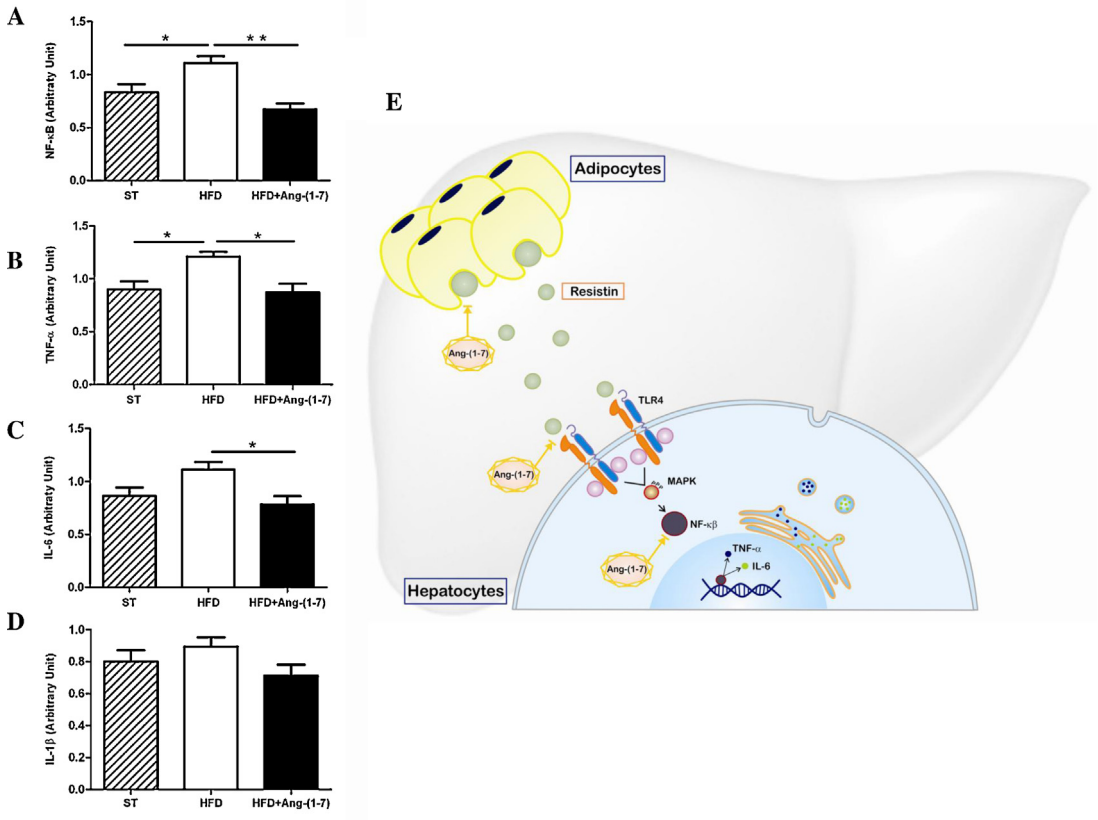

Fig. 3. Liver mRNA expression performed by qRT-PCR analyses. (A) Expression of NF-κB. (B) Expression of TNF-α. (C) Expression of IL-6. (D) Expression of IL-1β. (E) Illustration of Ang-(1–7)/resistin/TLR4/MAPK/NF-κB signaling-pathway. Data are presented as means ± SE. Statistically significant differences between the groups are indicated as $^{*}P < 0.05$ and $^{**}P < 0.01$ in comparison to the HFD group.

Oh et al. recently showed that captopril (ACE inhibitor) intake decreases body weight gain via Angiotensin-(1–7) [14]. This alteration was associated with Mas receptor mRNA increased expression [14]. Additionally, a previous study with DOCA salt-induced hypertension transgenic rats, that presents an overexpression of Ang-(1–7) in the circulation, also showed an increase in heart Ang-(1–7) accompanied by a decrease in ACE mRNA expression [17]. These data support our hypothesis of a modulatory role for Ang-(1–7) in the ACE/ACE2 ratio. Another possibility is that the improved metabolic profile by itself, with lower lipid content and enhanced glucose metabolism, was able to increase ACE2 and decrease ACE expression.

A previous report revealed that Ang II treatment increases adipocytes secretion of resistin [7]. Resistin has been also associated with the inflammatory state of chronic liver disease [25] and modulates the synthesis and secretion of key proinflammatory cytokines such as TNF-α and IL-6 [24]. The molecular mechanisms involved in the inflammatory response of resistin are still unclear, however a recent report indicated that resistin could compete with LPS for TLR4 [9]. Additionally a recent investigation reported the contribution of central resistin overexposure to induction of insulin resistance through TLR4 and activation of MAPK pathway [1]. In our study, we showed decreased TLR4 mRNA expression in liver of HFD + Ang-(1–7) rats associated with low phosphorylation of MAPK. This fact is important once resistin-TLR4 signaling in the hypothalamus leads to the activation of MAPK pathway promoting overall inflammation [1].

It is known that MAPK activation initiates the downstream induction of transcription factors such as NF-κB, which is an essential regulator of the expression of numerous genes involved in the function and development of the immune system and in inflammatory responses [22]. Activated NF-κB is the major regulator, facilitating the synthesis of several different injury-responsive cytokines in neurons, adipose tissue and liver [2,22,24]. Previous studies showed an elevated level of NF-κB in the adipose tissue of rats with increased levels of resistin [15,25]. In this study, oral treatment with Ang-(1–7) reduced TNF-α and IL-6 through inhibition of NF-κB.

5. Conclusion

In summary the present study showed that Ang-(1–7) oral treatment in rats fed high-fat feed prevent obesity and the decrease of several liver proinflammatory cytokines by down-regulating the resistin/TLR4/NF-κB pathway. Thus, our findings suggest that ACE2/Ang-(1–7)/Mas axis might be a novel therapeutic agent for the prevention and treatment of obesity-related liver disorders.

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